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Generation of Restriction Map of *Enterococcus faecalis* OG1 and Investigation of Growth Requirements and Regions Encoding Biosynthetic Function

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A defined synthetic medium was used to determine the amino acid requirements of *Enterococcus faecalis* OG1 and to demonstrate the absence of a requirement for exogenous purines or pyrimidines. Genomic libraries prepared from strain OG1 were transduced into *Escherichia coli* auxotrophic mutants, and cloned DNAs which complemented *pyrC*, *pyrD*, *purF*, *purL*, and *guaAB* mutations were identified. These and other cloned DNAs with known functions were localized on a restriction map of OG1 which was generated with *Sfi*I (5 fragments), *Asc*I (9 fragments), and *Not*I (15 fragments); the size of the OG1 chromosome was revised from a previous estimate of ~2,750 kb to 2,825 kb. The synthetic medium and the restriction map should be useful for studying enterococcal metabolic functions and the relationships between chromosomally encoded genes.

Enterococci are variably classified as the second to third most common organisms found in hospital-acquired infections, and 85 to 95% of these isolates are *Enterococcus faecalis* (16, 25). While a number of studies have addressed the clinical problems of *E. faecalis*, as well as its antibiotic resistance, plasmids, and transposons (5, 6, 15–17), there is relatively little known about the organism's more intrinsic properties, such as the chromosomal organization, the physical relationship of its genes, or its metabolic and biosynthetic capabilities. Indeed, there is little in the recent literature that addresses the growth requirements of enterococci. Because of the increasing clinical importance of these organisms, we have initiated studies aimed at increasing our fundamental understanding and knowledge of *E. faecalis*. Toward this goal, we have investigated growth requirements of enterococci, generated a restriction map of the well-known strain OG1, identified clones containing genes with biosynthetic functions, and located these and other described genes on our chromosomal map.

MATERIALS AND METHODS

Bacterial strains and plasmids. A derivative of *E. faecalis* OG1 resistant to rifampin and fusidic acid was used as the source of enterococcal chromosomal DNA (18). *Escherichia coli* K-12 strain MG1655 was the parent strain for *E. coli* auxotrophic mutants which had been previously generated by transposon mutagenesis using Tn*10*dCamMCS or Tn*10*dCamNS in the laboratory of one of us (G.M.W.) and at the Advanced Bacterial Genetics course at Cold Spring Harbor Laboratory (7, 10). The cosmid vector pLAFRx is a derivative of pLAFR which contains *oriT* of RK2 (10). INY3000Ω, INY3039Ω, INY3040Ω, INY3044Ω, and INY3048Ω (29) and INY1200Ω (28) contain insertions of Tn*916* or Tn*925* into strain OG1SSp; we have compared the restriction endonuclease digestion patterns of OG1, OG1RF, and OG1SSp and found them to be identical (unpublished). Strains of *E. faecalis* used for the determination of growth requirements include OG1RF (18), JH2-2 (30), ATCC 29212, and 20 clinical isolates including antibiotic-resistant *E. faecalis* previously described (19, 20). An *E. coli* strain with part of the gelatinase gene (*gel*) from OG1 was kindly provided by D. Clewell (27). *E. coli* S9 containing a probe for an autolysin gene from an *E. faecalis* isolate (2) cloned into pTZ18R was kindly provided by G. Bellemare. Other probes used were *phoS* (alkaline phosphatase) (23), *nox* (NADH oxidase), *npr* (NADH peroxidase), and *lpd* (lipoamide dehydrogenase) from *Streptococcus* (*Enterococcus*) *faecalis* 10C1 (ATCC 11700) (22). The plasmid pAM1016 (supplied in XL1-Blue by D. Clewell) was used as a probe for *tetM* to verify insertions of Tn*916* and Tn*925*.

Media and determination of growth requirements of enterococcal strains. For growth of *E. coli* prototrophs and for complementation, Davis minimal medium (7 g of K_2HPO_4, 2 g of KH_2PO_4, 0.5 g of sodium citrate, 0.1 g of $MgSO_4$, 1 g of NH_4SO_4 [all per liter]) (Difco Laboratories, Detroit, Mich.) or M63 salts (13), each supplemented with 100 μg of thiamine per ml, 0.1% glucose (0.2% for M63) and 1.5% Bacto Agar (Difco), was used. To define some of the individual growth requirements of OG1RF and other enterococci, a complete synthetic agar medium was made by using Davis minimal medium plus agar, glucose, and thiamine as described above. To this was added adenosine, guanosine, thymine, uracil, and 20 amino acids (each at a final concentration of 20 μg/ml); additional vitamins were biotin, calcium, pantothenic acid, and pyridoxine (each at 20 μg/ml), nicotinic acid and riboflavin (each at 2 μg/ml), and folic acid (0.2 μg/ml). These concentrations were arbitrarily chosen, using concentrations known to be adequate for growth of *E. coli* auxotrophs as a rough guide (7). Following demonstration of growth by OG1RF and other enterococci on this agar medium (referred to as enterococcal complete synthetic medi-

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um), individual components were then omitted to determine which were required for growth. Cultures were streaked for single colonies, and the resulting growth was assessed after 24 to 48 h of incubation at 37°C.

For growth in broth, the enterococcal complete synthetic medium described above but without agar was used. Enterococcal strains were grown overnight in brain heart infusion broth (Difco Laboratories), harvested, washed once in 0.9% NaCl and then added to the defined medium in a final inoculum of 10^6 to 10^7 CFU/ml.

Preparation and amplification of enterococcal genomic libraries. Enterococcal genomic DNA was prepared by the method of Hull et al. (11) with modifications to achieve lysis of enterococci. Briefly, OG1 was grown overnight in 200 ml of brain heart infusion broth, harvested, washed once in TE buffer (0.01 M Tris, 0.001 M EDTA; pH 8), and resuspended in 2 ml of 25% sucrose as described by Hull et al. Lysozyme (0.1 ml of 40 μg/ml) plus mutanolysin (~100 U) were then added. After 5 min of incubation on ice, 50 μl of proteinase K (25 μg/ml) was added. This was followed by the addition of 1.5 ml of EDTA (0.5 M, pH 8.0) and incubation on ice for 5 min. Cells were lysed by the addition of 1.5 to 2.0 ml of Sarkosyl (20%) with incubation on ice until lysis was complete. The lysate was incubated an additional 30 min at 37°C in a water bath followed by overnight incubation at 50°C after being covered with aluminum foil. The remainder of the procedure and preparation of the cesium chloride gradients were as described by Hull et al. (11).

Following removal from cesium chloride, the resulting DNA was dialyzed against TE buffer for 48 h and then partially digested with *Eco*RI, *Hin*dIII, or *Bam*HI (24). The partially digested DNA was size fractionated in a sucrose density gradient with selection of fractions containing fragments of 18 to 28 kb. This DNA was then ligated into pLAFRx which had been precipitated from cesium chloride gradients, dialyzed, and then digested with the appropriate enzyme. The ligated mixture was packaged in vitro using Packagene (Promega, Madison, Wis.), transduced into *E. coli* LE392, and plated onto LB agar (24) containing tetracycline (12.5 μg/ml) (LB-TET). The presence of cloned inserts was determined by restriction endonuclease digestion of DNA from individual colonies (see below). The plasmid (cosmid) names are derived from the name assigned to the clone, e.g., pKV2 comes from clone KV2. Following screening of a number of individual colonies, the libraries were amplified and stored as lambda lysates.

Minipreparations, plasmid digestions, and transformation of *E. coli*. Isolation of plasmid DNA from desired clones was performed by a slightly modified alkaline sodium dodecyl sulfate (SDS) protocol (3). The plasmid preparations were analyzed after restriction endonuclease digestions with 0.5% agarose gels in 1× TBE (10× TBE is 0.89 M Tris, 0.25 M EDTA, and 0.89 M boric acid). Preparation of competent cells and transformations of plasmid DNA were performed by the procedure of Chung et al. (4).

Complementation. Chloramphenicol-resistant auxotrophic mutants of *E. coli* MG1655 previously generated by insertion of transposition-deficient derivatives of Tn*10* were grown overnight in LB agar, and 100 μl was plated on M63 minimal agar incubated at 37°C overnight. On the basis of the number of phenotypic revertants, 1 to 10 μl of the original overnight culture was added to tubes containing 90 μl of TMG (10 mM Tris, 5 mM $MgSO_4 \cdot 7\ H_2O$, 0.01% gelatin; pH 7.4) and 10 μl of the lambda lysates of the amplified libraries; this mixture was incubated for 10 to 20 min at room temperature, and then the entire mixture was plated onto M63 minimal agar containing glucose and thiamine (as described earlier), and 25 μg of chloramphenicol per ml. Plasmid DNA was then prepared from individual colonies (potential complementing clones) which grew on this minimal medium, transformed into the respective auxotrophs, and plated on LB-TET to select for pLAFRx-containing transformants. Tetracycline-resistant colonies were then scored for the ability to grow on minimal medium; those colonies which grew were again purified and subjected to another round of transformation and selection on LB-TET, followed by scoring for growth on minimal medium. Those derivatives which grew were considered complementing clones.

Mapping. Preparation of agarose plugs of OG1 and single and double digestions with *Sfi*I, *Asc*I, *Not*I, and in some instances, *Sma*I were performed as previously described (14, 19). Partial digestions were performed with 5 U of *Not*I followed by incubation at 37°C for 5, 10, 15, 20, 25, 30, and 60 min. Pulsed-field gel electrophoresis (PFGE) was performed with 1.6% SeaKem GOLD agarose (GTG grade; FMC Bioproducts, Rockland, Maine) in 0.5× TBE or 1% FastLane agarose (FMC Bioproducts) in 0.25× TBE, using a CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, Calif.), and varying the conditions, depending on which size range was being examined. Gels were stained in ethidium bromide, exposed to UV light (254 nm) for 80 to 90 s, and photographed. The gels were then immersed in 0.4 N NaOH for 15 to 20 min, and the DNA in gels was transferred to HyBond-N filters (Amersham Corporation, Arlington Heights, Ill.) with 0.4 N NaOH. After 24 to 36 h of transfer, the membranes were neutralized for 5 min in 0.5 M Tris HCl (pH 7.0), rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), dried, and baked for 1 to 2 h at 85°C. Filters were hybridized with radiolabeled plasmid DNA from clones from the libraries and to probes for known genes, and these results were used to determine the relationships between the fragments of OG1RF. The *E. coli* plasmids showed no hybridization to OG1 DNA. INY strains containing copies of Tn*916* or Tn*925* were examined for a change in fragment size and by hybridization to a probe for *tetM*. For rRNA genes, the 5S and 16S plus 23S probes from *E. coli* MRE 600 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) were used.

To use restriction fragments for hybridization, restriction endonuclease-digested DNA was electrophoresed in 1.6% SeaKem GOLD agarose or 1.2% SeaPlaque GTG grade agarose (FMC Bioproducts) in 0.5× TBE. Individual fragments were excised, incubated for 4 to 6 h in 1 ml of sterile distilled H_2O at 37°C, digested with a restriction endonuclease with many recognition sites and the appropriate restriction buffer. Following incubation overnight at 37°C, the DNA was then eluted by Gene Clean (Bio 101, La Jolla, Calif.) in a total volume of 10 to 20 μl and used for radiolabeling with [^{32}P]dCTP, using the random priming DNA labeling kit (Boehringer Mannheim Biochemicals). Subclones of specific fragments were prepared following in-gel digestion with *Bam*HI plus *Hin*dIII as described above, followed by ligation into the *Bam*HI-*Hin*dIII-digested pUC18. Ligated DNA was then transformed into competent JM105 cells.

Membranes were prehybridized overnight in 25 ml containing 5× SSPE, 5× Denhardt's solution, 0.5% SDS, 50% formamide, and 200 μg of heat-denatured calf thymus DNA per ml per the recommendations of Amersham. Radiolabeled DNA probes (~10^7 cpm per filter) and calf thymus DNA (200 μg/ml) were mixed together, boiled for 5 to 7 min, cooled on ice, and then added to the bags containing the membrane and

prehybridization fluid. Hybridization was carried out at 42°C. Membranes were washed twice for 10 min with 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–0.1% SDS at room temperature, followed by one 10-min wash at 65°C with 1× SSPE–0.1% SDS, and one 15-min wash with 0.1× SSPE–0.1% SDS. Membranes were air dried and then exposed to Kodak XAR X-ray film at −70°C. After autoradiography, filters were stripped by pouring hot 0.1% SDS over the membrane and allowing it to cool to room temperature; these membranes were then exposed to X-ray film to verify adequacy of the stripping.

RESULTS

Determination of the growth requirements of OG1. Strain OG1 did not grow on Davis minimal agar plus thiamine and glucose but grew well when this agar was supplemented with six other vitamins plus 20 amino acids. Adenosine, guanosine, uracil, and thymine were not required for growth on this agar medium. Because the literature cites enterococci as requiring multiple vitamins for growth (8, 12, 21), no effort was made to delete vitamins from the medium.

Omitting individual amino acids from this medium revealed that all 23 *E. faecalis* strains, including OG1, require histidine, isoleucine, methionine, and tryptophan for growth. Omission of arginine, glutamate, glycine, leucine, or valine resulted in no growth for some strains (e.g., JH2-2 and ATCC 29212) and poor growth for others (e.g., OG1). Omission of cysteine, serine, or threonine had no obvious effect on growth of some organisms (e.g., JH2-2 and OG1RF) but led to somewhat poorer growth by others (e.g., ATCC 29212). No visible difference in growth of any of the isolates was observed following omission of alanine, asparagine, aspartate, glutamine, lysine, phenylalanine, proline, or tyrosine.

We next tested the ability of enterococci to grow on minimal agar medium with the above-listed vitamins and glucose plus combinations of the amino acids that affected growth. Pinpoint colonies were seen at 24 h following streaking upon this medium containing arginine, glutamate, glycine, histidine, isoleucine, leucine, methionine, tryptophan, and valine (20 μg/ml [each]) (the nine required or almost required amino acids); this was called our basal agar medium. The addition of serine, threonine, and cysteine to the other nine amino acids generated better growth at 48 h for OG1 and most of the other *E. faecalis* strains. When tyrosine or phenylalanine was added, growth was indistinguishable from that on the medium with all 20 amino acids.

Ten strains were tested in the broth version of our complete synthetic medium and most showed growth similar to that seen with the agar medium. In summary, OG1 and most of the other enterococci were prototrophic for purines and pyrimidines and eight amino acids (Ala, Asn, Asp, Gln, Lys, Phe, Pro, and Tyr), and auxotrophic (or almost so) for Arg, Glu, Gly, His, Ile, Leu, Met, Trp, and Val. The addition of Cys, Ser, plus Thr to the nine most required amino acids stimulated growth.

Preparation of OG1 genomic libraries in pLAFRx and complementation. Following construction of the genomic libraries and selection on tetracycline-containing agar, restriction endonuclease digestion of plasmid DNA from tetracycline-resistant colonies revealed that about 25% of the colonies from the libraries constructed with *Eco*RI and *Hin*dIII libraries had inserts, while 12.5% of the Tet^r colonies from the library constructed with *Bam*HI had inserts. To identify some of the genes involved in biosynthesis pathways, we transduced the OG1 DNA libraries into *E. coli* auxotrophic mutants and plated these mixtures on minimal medium. Potential complementing clones were retested with two rounds of transformation and selection for tetracycline resistance followed by independent scoring for prototrophic growth. Complementation of five of the 22 *E. coli* auxotrophs tested was seen, namely, *guaAB*, *purF*, *purL*, *pyrC*, and *pyrD* mutants. No complementation was seen with *argECBH*, *argG*, *aroA*, *asp*, *carAB*, *cysCDHIJ*, *his*, *ilv*, *leu*, *lysA*, *metE*, *metJBLF*, *nadC*, *pan*, *serA*, *thr*, and *trp* mutants.

Generation of a restriction map of OG1. We previously estimated the size of the OG1 chromosome using *Not*I, *Sfi*I, and *Sma*I as 2,750 to 2,761 kb (14). The revised size estimate after mapping the chromosome is 2,825 kb (Fig. 1). The sizes of fragments represent average values generated from those gels where appropriate size markers were well separated with subsequent minor adjustments for a best fit of all the data. The largest change was 10 kb, changing *Sfi*I-A from 660 to 670 kb (1.5%). The largest percent change was 4% (a 2-kb change in *Asc*I-D from 46 to 48 kb). Most changes were ≤2%, which is within our routine variation from gel to gel. Three *Not*I fragments not identified previously were as follows: (i) a 6-kb *Not*I fragment (*Not*I-K); (ii) *Not*I-J (173 kb), not previously separated from *Not*I-G (180 kb) (Fig. 2); and (iii) *Not*I-I (27 kb), which has still not been resolved from *Not*I-H (28 kb) but whose presence was shown by partial digestions (see below).

Several approaches were used to generate the restriction map. (i) Forty cloned regions and genes were hybridized to digestion fragments digested with one or two enzymes to relate fragments generated by one enzyme to those generated by other enzymes (see Table 2 for a summary); a gel and two autoradiograms are also shown in Fig. 3. (ii) Restriction fragments were isolated from gels following PFGE and used as probes. (iii) A combination of complete digestion with one enzyme and partial digestion with another enzyme followed by hybridization was used to order some fragments (one example shown in Fig. 3). (iv) Four transposon insertions of Tn*916* and two of Tn*925* into an OG1 derivative were also used (see Table 2).

In generating the physical map of OG1, the 0 point was arbitrarily set at one end of the *Not*I-A fragment. Clone KV29 is a double linking clone between *Not*I-A and *Not*I-O and between *Asc*I-A and *Asc*I-I. This cloned DNA also hybridized to the *Sfi*I-A fragment, establishing a relationship between fragments generated by these three enzymes. Digestion of pKV29 with *Not*I and *Asc*I showed a distance of ~2.4 kb between the sites for these enzymes. *Not*I-*Sfi*I digestion plus hybridization to pKV21 showed that *Not*I-A was not digested by *Sfi*I and that a new *Not*I-*Sfi*I fragment of 66 kb was generated. Hybridization of pKV29 to *Asc*I-*Sfi*I double digestion products showed fragments of 68 and ~214 kb (*Asc*I-A). The fact that the *Asc*I-*Sfi*I fragment (68 kb) is larger than the *Not*I-*Sfi*I fragment (66 kb) indicates that the *Asc*I site between *Asc*I-A and *Asc*I-O is positioned past the *Not*I site, in a clockwise direction from the *Sfi*I site.

The results obtained with the other probes are shown in Table 1 and Fig. 1. Results obtained with specific fragments and with double and partial digestions are summarized below. The *Not*I A fragment hybridized to itself, to *Asc*I-A, and to *Sfi*I-A. *Asc*I-A hybridized to itself, to *Sfi*I-A, and to *Not*I-A and weakly hybridized to *Not*I-B. These results suggest that *Asc*I-A overlaps *Not*I-B, and indeed *Not*I-B contains an *Asc*I site. *Sfi*I-A hybridized to *Not*I-A, *Not*I-B, *Not*I-C, and *Not*I-D and weakly hybridized to *Not*I-O.

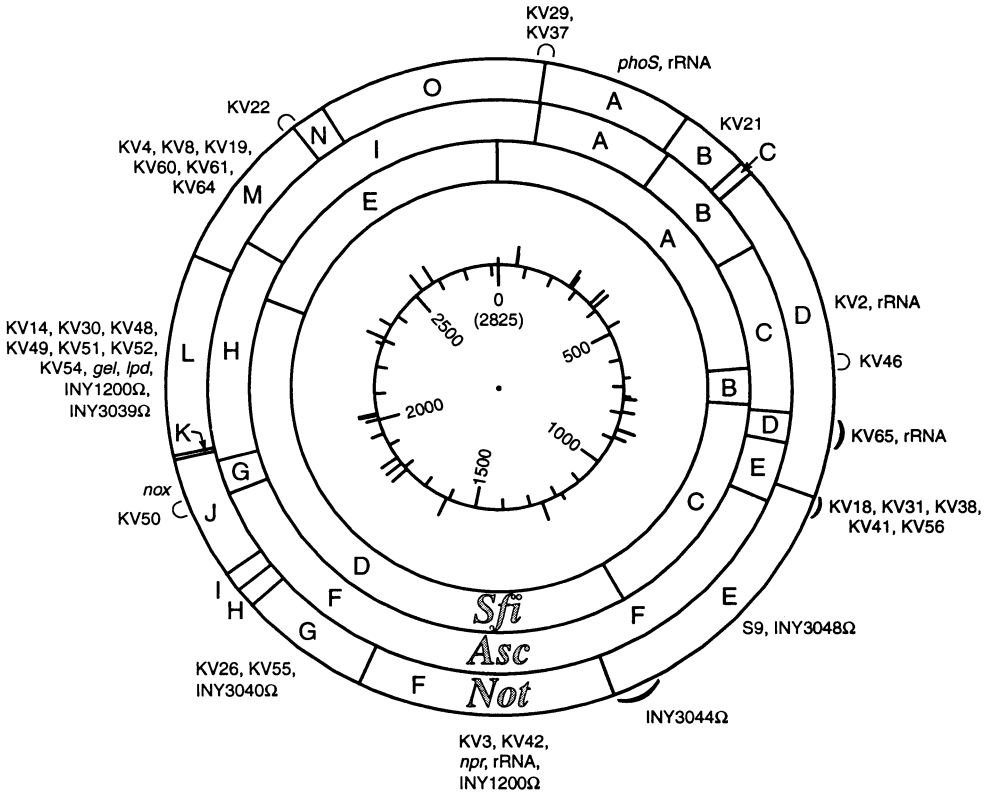

FIG. 1. Restriction map of OG1 using *Sfi*I, *Asc*I, and *Not*I. Fragment sizes are given in Table 1. The semicircles (∩) indicate linking clones. The heavier arcs (⁀) were added in some locations to clarify which subfragments contained certain cloned DNAs or insertions.

*Sfi*I-A also hybridized to the *Asc*I-A, *Asc*I-B, *Asc*I-C, and *Asc*I-D fragments and weakly hybridized to *Asc*I-I; hybridization to *Asc*I-D likely results from the presence of rRNA genes in these fragments (Table 2). *Not*I-C hybridized to *Asc*I-B and *Sfi*I-A, was not digested by *Asc*I, and did not hybridize to *Asc*I-A, confirming its position past *Not*I-B. *Asc*I-B hybridized only to *Not*I-B, *Not*I-C, and *Not*I-D, indicating that there are no other fragments between the *Not*I-B and *Not*I-C fragments. Hybridization of pKV21 to *Asc*I-*Not*I-digested DNA showed that ~9 kb was cleaved off *Not*I-B by *Asc*I digestion, as predicted by the map. By using complete *Asc*I digestion plus partial *Not*I digestion, fragments of ~86 kb (*Not*I-B cleaved by *Asc*I) and 105 kb hybridized to pKV21, confirming that a 19-kb fragment (*Not*I-C) is adjacent to *Not*I-B (86 kb plus 19 kb = 105 kb) (data not shown).

Double digestion with *Asc*I and *Sfi*I generated a 193-kb fragment (from *Sfi*I-A and *Asc*I-C) which hybridized to pKV2. This size fragment indicates that there can be no other *Asc*I fragment between *Asc*I-B and *Asc*I-C. With *Not*I-and-*Sfi*I double digestion, pKV46 hybridized to a 68-kb fragment (*Sfi*I-B) and to a 282-kb fragment that also hybridized to pKV2.

*Not*I-D hybridized to *Asc*I-B, -C, -D, and -E fragments and to the *Sfi*I-A, -B, and -C fragments. *Asc*I-D was initially thought to be a single fragment (Fig. 2); however, under different electrophoresis conditions, two bands are clearly visible (*Asc*I-D [48 kb] and *Asc*I-G [54 kb]). *Asc*I-D is placed past *Asc*I-C, since pKV2 hybridized to a 193-kb *Asc*I-*Sfi*I fragment which is not compatible with *Asc*I-D (48 kb) being positioned between *Asc*I-B and *Asc*I-C. *Asc*I-D hybridized not only to itself but to the *Asc*I-C and *Asc*I-A fragments and to several fragments in the *Not*I and *Sfi*I digestions. A subclone of this fragment, KV65, hybridized only to *Asc*I-D, *Not*I-D, and *Sfi*I-C. Another subclone, KV66, hybridized to the same fragments that hybridized to the rRNA probes (Table 2).

A *Not*I-*Sfi*I double digestion fragment of approximately 124 kb (from the start of the *Sfi*I-C to the start of *Not*I-E) hybridized to *Asc*I-C, *Asc*I-D, *Asc*I-E, to *Not*I-D, and to *Sfi*I-A, *Sfi*I-B, and *Sfi*I-C, although only hybridization to *Asc*I-D, *Asc*I-E, *Not*I-D, and *Sfi*I-C was predicted. Other data showed that this region contains rRNA genes. Even with the cross-hybridization, these data suggest that the *Asc*I-E fragment (which does not hybridize to rRNA genes) follows *Asc*I-D and that *Sfi*I-C follows *Sfi*I-B, conclusions supported by results using several clones (Table 2). Hybridization of pKV31 to *Not*I-E, *Asc*I-E, and *Sfi*I-C indicated that *Not*I-E follows *Not*I-D. pKV31 hybridized to a 29-kb *Not*I-*Asc*I fragment, indicating that *Asc*I-E ends approximately 29 kb after the beginning of *Not*I-E.

Based on results with clone S9 and with the Tn*916* insertion of INY3048Ω, *Asc*I-F was placed next to *Asc*I-E. DNA from S9 also hybridized to a 316-kb *Not*I-*Sfi*I fragment (extending from the start of *Not*I-E to the end of *Sfi*I-C), to a 286-kb *Asc*I-*Sfi*I fragment (from the start of *Asc*I-F to the end of *Sfi*I-C), and a 363-kb *Not*I-*Asc*I fragment (from the start of *Asc*I-F to the end of *Not*I-E).

Hybridization with a number of clones showed that *Not*I fragments F, G, H, and I are contained within the *Asc*I-F and *Sfi*I-D fragments (Table 2). In order to establish the relative order of these fragments, partial digestions and hybridization

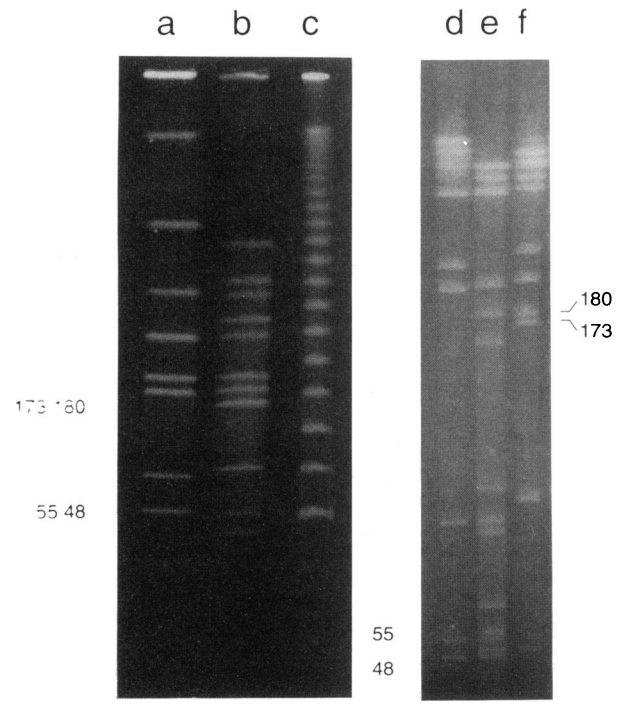

FIG. 2. PFGE of restriction endonuclease-digested OG1 chromosomal DNA. Lanes a and d contain *Asc*I-digested DNA; in lane a, *Asc*I-G (55 kb) and *Asc*I-D (48 kb) migrate as a single fragment but are clearly separated in lane d. Lanes b and f contain *Not*I-digested DNA; in lane b, *Not*I-J (173 kb) and *Not*I-G (180 kb) migrate as a single fragment but are clearly separated in lane f. Lane c contains lambda concatamers; lane e contains *Asc*I-*Not*I doubly digested DNA. The sizes are given in kilobases.

TABLE 1. Sizes of restriction digestion fragments

*Not*I fragment	Size (kb)	*Asc*I fragment	Size (kb)	*Sfi*I fragment	Size (kb)
A	207	A	214	A	670
B	96	B	195	B	68
C	19	C	270	C	440
D	474	D	48	D	1,107
E	393	E	97	E	540
F	355	F	1,055		
G	180	G	55		
H	28	H	356		
I	27	I	535		
J	173				
K	6				
L	282				
M	223				
N	48				
O	314				
Total	2,825		2,825		2,825

were performed. One of the most helpful experiments is shown in the autoradiogram in Fig. 4. Complete *Asc*I digestion generated fragments F and G (~1055 and 55 kb) that hybridized to pKV50, and complete *Not*I-*Asc*I digestion generated fragments of 102 and 55 kb (*Asc*I-G) that hybridized to this clone. Complete *Asc*I digestion plus partial *Not*I digestion generated 55- (*Asc*I-G), 102- (the *Not*I-*Asc*I complete digestion product), 129-, 155-, and 335-kb fragments plus larger fragments that hybridized to pKV50. The presence of both 129- and 155-kb fragments indicate that there are two ~27-kb fragments, although only one could be seen on gels. The fragment sizes following hybridization to pKV50 showed that one fragment is slightly larger (~28 kb). The fragment adjacent and moving counterclockwise from *Not*I-J is the smaller one, i.e., *Not*I-I (27 kb), since 129 kb minus 102 kb (the *Not*I-*Asc*I subfragment of *Not*I-J) equals 27 kb. *Not*I-H is placed adjacent to *Not*I-I, since 155 kb minus 127 kb equals 28 kb. The next fragment seen in the partial digestions is ~335 kb, which indicates that *Not*I-G (180 kb) is next to *Not*I-H (155 kb + 180 kb = 335 kb). Since *Not*I-F is the only other fragment in this region, it must be next to *Not*I-G. Two larger fragments were also seen on this autoradiogram but were obscured by a large black smear. Autoradiograms derived from other gels showed the 55- (*Asc*I-G), 102-, 129-, 155-, and 335-kb fragments plus additional fragments of ~1,055 kb (*Asc*I-F) and of ~700 kb,

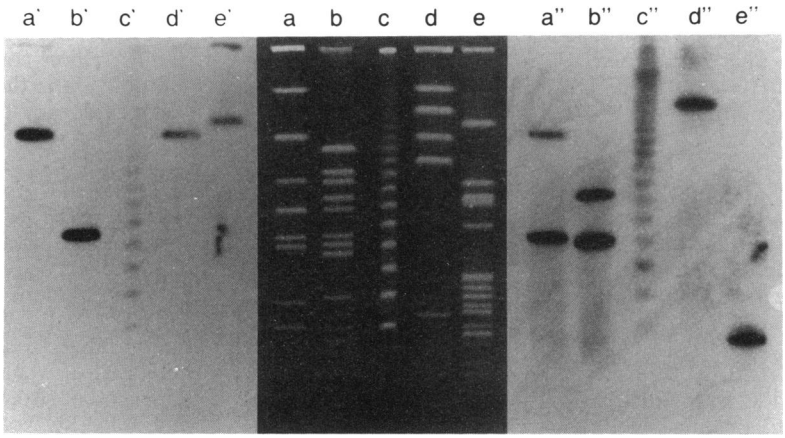

FIG. 3. PFGE and autoradiograms of digested OG1 DNA. The autoradiogram on the left (using clone KV4 as a probe) was prepared with the gel shown in the center panel, while the autoradiogram on the right (using clone KV37 as a probe) was prepared from a different gel. DNA was digested with *Asc*I (lanes a, a', and a''), *Not*I (lanes b, b', and b''), *Sfi*I (lanes d, d', and d''), and *Sma*I (lanes e, e', and e''). Lambda concatamers are in lanes c, c', and c''. The following fragments are visible: *Asc*I-I (lane a'), *Not*I-M (lane b'), *Sfi*I-E (lane d), *Asc*I-I and *Asc*I-A (lane a''), *Not*I-O and *Not*I-A (lane b''), and *Sfi*I-A (lane d'). The *Sma*I fragments were not named.

TABLE 2. OG1 hybridization summary

Probe(s)	Fragment or insertion hybridizing to probe			Relevant information
	*Asc*I	*Not*I	*Sfi*I	
Clones				
KV2	C	D	A	
KV3, KV42	F	F	D	
KV4, KV8, KV19, KV60, KV61, KV64	I	M	E	
KV14, KV30, KV54	H	L	D	
KV18, KV31, KV38, KV41, KV56	E	E	C	
KV21	B	B	A	
KV22	I	M, N	E	
KV26, KV55	F	G	D	
KV29, KV37	I, A	O, A	A	
KV40, KV43, KV57	F	G/J[a]	D	
KV46	C	D	A, B	*guaAB*
KV48	H	L	D	*pyrC*
KV49	H	L	D	*pyrD*
KV50	F, G	J	D	
KV51	H	L	D	*purL*
KV52	H	L	D	*purF*
KV65	D	D	C	Subclone from *Asc*I-D
KV66	A, C, D, F	A, D, F	A, C, D	Subclone from *Asc*I-D
S9	F	E	C	Autolysin
Genes				
phoS	A	A	A	Alkaline phosphatase
nox	G	J	D	NADH oxidase
npr	F	F	D	NADH peroxidase
lpd	H	L	D	Lipoamide dehydrogenase
gel	H	L	D	Gelatinase
rRNA (5S, 16S + 23S)	A, C, D, F	A, D, F	A, C, D	
Transposon insertions				
INY3039Ω	H	L	D	Tn*916* (29)
INY3040Ω	F	G	D	Tn*916* (29)
INY3044Ω	F	E	D	Tn*916* (29)
INY3048Ω	F	E	C	Tn*916* (29)
INY1200Ω	H, F	F, L	D	Tn*925* (2 insertions) (28)

[a] Determination of which of these two closely migrating fragments hybridized to these clones was not made.

which confirms that *Not*I-F (355 kb) must be next to *Not*I-G (355 + 335 = 690 kb) (data not shown). Similar experiments using KV26 and KV3 also yielded results consistent with the map shown in Fig. 1. Fragments generated included fragments of 1,055 kb (*Asc*I-F) and 180 kb (*Not*I-G), seen in the complete digestions with *Asc*I and *Not*I, respectively, and fragments of 208 kb (180 kb [*Not*I-G] plus 28 kb [*Not*I-H]), 235 kb (208 kb plus 27 kb [*Not*I-I]), 335 kb (235 kb + 100 kb derived from *Asc*I digestion of *Not*I-J), and 537 kb (180 kb [*Not*I-G] plus 357 kb [*Not*I-F]), and fragments of approximately 565 (537 kb plus 28 kb [*Not*I-H] = 565 kb) and 592 kb (565 kb + 27 kb [*Not*I-I] = 592 kb).

Three additional fragments, *Not*I-K, *Not*I-L, and *Asc*I-H, were found to be associated with the *Sfi*I-D fragment (Table 2). pKV48 hybridized to a *Not*I-*Sfi*I fragment of 261 kb, indicating that *Sfi*I-D stops ~21 kb from the end of the 282-kb *Not*I-L fragment. Hybridization of KV48 to *Not*I-*Asc*I double digestions showed that *Not*I-L has no *Asc*I site, and thus the *Asc*I-H fragment begins before the *Not*I-L fragment. The *Not*I-K fragment (6 kb) was not seen on most pulsed-field gels and was best seen on conventional agarose gels. When this fragment was used as a probe, hybridization occurred to most fragments, presumably because of contamination of the 6-kb region of the gel by sheared remnants of other fragments. The *Not*I-K fragment was then digested with *Eco*RI, and bands were excised to use as probes.

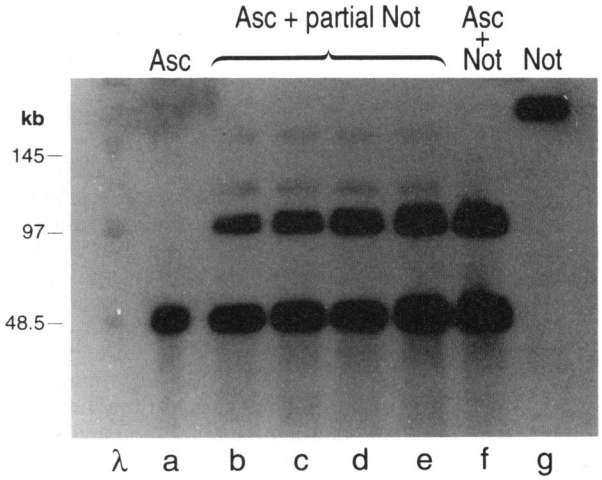

FIG. 4. Autoradiogram prepared from PFGE of digested DNA, using pKV50 as a probe. Lane a contains completely *Asc*I-digested DNA (only *Asc*I-G of 55 kb is shown); lane g, completely *Not*I-digested DNA; and lane f, completely *Asc*I-*Not*I-digested DNA. Lanes b to e show completely *Asc*I-digested DNA that was then partially digested by *Not*I. Lambda concatamers were visualized by using radiolabeled lambda DNA as a probe.

Although there was still some faint background hybridization to multiple fragments, the strongest hybridization was to *Not*I-K, *Asc*I-H, and *Sfi*I-D, which places *Not*I-K between *Not*I-J and *Not*I-L.

Establishing the order of the fragments following *Not*I-L, *Asc*I-H, and *Sfi*I-D required use of *Sma*I digestion fragments. Clones KV4 (from *Not*I-M), KV22 (from *Not*I-M and -N) and KV54 (from *Not*I-L), all hybridized to a *Sma*I fragment of approximately 625 kb, showing the proximity of these three *Not*I fragments. The positioning of *Not*I-N between *Not*I-M and *Not*I-O is supported by the observation that *Not*I-M hybridized to *Not*I-*Asc*I double digestion fragments of ~171 and ~52 kb, as predicted by the position of the *Asc*I site between *Asc*I-H and *Asc*I-I. If *Not*I-N were on the other side of *Not*I-M, then *Not*I-*Asc*I double digestions would not generate these fragments, nor would pKV22 hybridize to *Asc*I-I. *Sfi*I-*Asc*I double digestions generated a fragment of ~467 kb (from the start of the *Asc*I-I fragment to the end of the *Sfi*I-E fragment) which hybridized to pKV22. Since pKV29 hybridized to a *Not*I-*Sfi*I fragment of ~66 kb (see above), we deduced that a *Not*I-*Sfi*I fragment of about ~248 kb should represent the other end of the *Not*I-O fragment. A 248-kb *Not*I-*Sfi*I fragment was used as a probe and hybridized to the *Not*I-O and *Sfi*I-E fragments, as predicted. As described above, *Not*I-O is linked to *Not*I-A and *Asc*I-I is linked to *Asc*I-A, thus completing the circular map.

DISCUSSION

It has been recognized for decades that enterococci have complex growth requirements and most studies with enterococci, even those of metabolic functions, have typically been performed in a complex medium or a semisynthetic medium such as casein hydrosylates (1, 8, 9, 12, 21, 26). Our observation that some but not all amino acids are required is consistent with observations made decades ago (12, 21), and in general, the amino acids we identified as required in the current study were also reported by earlier investigators as being required (12, 21). Major discrepancies were observed only for glycine and lysine (not required in this study but required in a previous work [12]) and, to a lesser extent, for serine and threonine (variably classified as stimulatory or required [12, 21]). Such differences may be due to the use of different strains, misidentification of *Streptococcus* (*Enterococcus*) *faecalis* in earlier studies, the use of different concentrations of amino acids, the use of D, L, and/or racemic mixtures of amino acids in previous studies, or the presence of trace contaminants. Although we classified only nine amino acids as being required or almost required for growth of OG1 and other enterococci, the combination of these nine amino acids supported less growth of *E. faecalis* than did all 20 amino acids together, perhaps because some of the nonessential amino acids may be interconverted to satisfy growth requirements. When serine, threonine, and cysteine were added to the nine most required amino acids, better growth of our *E. faecalis* strains were achieved. We did not test all possible combinations of amino acids, and it might be possible to support good growth with a different 12 amino acids than the ones we tested.

Although a number of amino acids were not required for growth, we were not able to recover complementing clones among the OG1 DNA libraries for the 14 amino acid auxotrophic mutants of *E. coli*, including seven which had mutations in biosynthesis pathways of amino acids not required for the growth of OG1. The inability to complement amino acid-requiring auxotrophs may be related to the fact that many of these biosynthesis genes in *E. coli* are present in operons and the mutants we used had been generated by transposon insertions which have polar effects. Alternatively, these enterococcal genes may not be expressed or may not be able to substitute for their *E. coli* counterparts, or may come from regions underrepresented in our DNA libraries.

In contrast, we were able to identify clones complementing five of the six *E. coli* mutants with insertions in purine and pyrimidine biosynthesis pathways. This result is consistent with the lack of a requirement of OG1 and other *E. faecalis* strains for purines and pyrimidines for growth. The ability to complement a larger number of defects in these pathways may also be related to the fact that the genes in these pathways are typically not in operons in *E. coli* so that the transposon insertions into one gene are less likely to interfere with expression of other genes in these pathways.

The localization of our clones on the restriction map of OG1 showed that four of the five functions complemented were from the *Not*I-L fragment. Two of these, *pyrC* and *pyrD*, are encoded on identical clones (KV48 and KV49). The *Not*I-L region seems to have been overrepresented in our DNA libraries with 10 of 40 clones or known genes coming from this fragment, which is only ~10 percent of the total chromosome. Other genes which were localized to this particular *Not*I fragment are *gel* and *lpd*; *nox* hybridized to the end of the *Not*I-J fragment closest to the *Not*I-L fragment. The other clones whose function was known (*phoS*, *npr*, *guaAB*, and the probe for an autolysin gene) were all from different regions of the chromosome.

The generation of the restriction map also allowed us to study the possible linkage of the four Tn*916* insertions present in INY3000Ω, a derivative of OG1SSp that is resistant to phage and which fails to function as a recipient in pheromone induced mating in broth (29). None of the four single insertions found in INY3000Ω, generated by transformation of chromosomal DNA from this derivative, yielded this phenotype. Insertions in INY3048Ω and INY3039Ω were >600 kb apart. INY3048Ω and INY3044Ω both have insertions in *Not*I-E and *Asc*I-F and could be closely linked even though they are on different *Sfi*I fragments (*Sfi*I-C and -D). The insertions of INY3039Ω and INY3040Ω are in *Not*I-G and *Not*I-I. Thus, of the four insertions known to have some effect on binding substance, only two have the potential of being closely linked.

In summary, we have identified some of the growth requirements of *E. faecalis* OG1 and other enterococci and defined a synthetic agar medium for these organisms. This medium should be valuable for general studies of enterococci, including metabolic studies, regulation of virulence and other functions, for simplifying protein purifications by reducing protein background levels, for studying transport, and for labeling of proteins and nucleic acids to high specific activities. The restriction map should be of general use to other investigators studying enterococci and should facilitate studies of possible linkages of other genes and of the possibility of chromosomal recombination, rearrangement, or mobilization between organisms.

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